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Short Communication

Further Characterization of Three *Yersinia enterocolitica* Strains with a Nalidixic Acid–Resistant Phenotype Isolated from Humans with Diarrhea

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Abstract

Antimicrobial-resistant bacteria pose a threat to public health. Three *Yersinia enterocolitica* strains cultured from patients presenting with diarrhea and resistant to nalidixic acid were studied. Target gene mutations in *gyrA* alone were identified as part of the genetic basis for this phenotype. Efflux activity was also noted, since the presence of the efflux pump inhibitor, phenylalanine-arginine-β-naphthylamide, increased susceptibility to nalidixic acid.

Introduction

Yersiniosis, a foodborne disease, is characterized by symptoms including diarrhea, fever, abdominal pain, and vomiting (Drummond *et al*., 2012). Fluoroquinolones (FQ) are used for the treatment of this infection, particularly in immunocompromised individuals (Capilla *et al*., 2004). In Gram-negative bacteria, three mechanisms of FQ resistance have been described including target gene mutation(s); reduction in drug accumulation; and plasmid-mediated quinolone resistance (PMQR). DNA gyrase and DNA topoisomerase IV are tetrameric enzymes and are the targets for quinolone drugs. Resistance has been attributed to chromosomal mutations in the corresponding subunit-encoding genes (*gyrA, gyrB* for DNA gyrase and/or *parC* and *parE* for topoisomerase IV) (Ruiz, 2003). Within these loci, the quinolone-resistance-determining region (QRDR) is a mutational hot spot (Fàbrega *et al*., 2010). Several mutations have already been identified in *gyrA* in *Yersinia enterocolitica*, the most common of which lead to amino acid substitutions at Ser-83 and Asp-87, conferring resistance to nalidixic acid (Sánchez-Céspedes *et al*., 2003; Capilla *et al*, 2004; Sihvonen *et al*., 2011).

Compared to other members of the Enterobacteriaceae, quinolone resistance in *Y. enterocolitica* is not commonly encountered. In this study, chromosomal- and plasmid-mediated resistance mechanisms were investigated in three nalidixic acid–resistant *Y. enterocolitica* recovered from humans in Switzerland. The data presented extend our understanding of this resistance type in these foodborne pathogens.

Materials and Methods

Bacterial strains and culture conditions

Three *Y. enterocolitica* strains (Table 1), isolated between 2006 and 2010 from humans in Switzerland, along with *Y. enterocolitica* 8081 and a reference-strain *Y. enterocolitica* ATCC™9610, were included in this study. All bacteria were subcultured and maintained as described previously (Murphy *et al*., 2010).

Antimicrobial susceptibility testing

Susceptibility testing was performed by disc diffusion, using a panel of antimicrobial agents including the following: amoxicillin-clavulanic acid 30 (10+20) μg, ampicillin 10 μg, cefoxitin 30 μg, ceftazidime 30 μg, cefpodoxime 10 μg, cefuroxime 30 μg, cephalothin 30 μg, ciprofloxacin 5 μg, gentamicin 10 μg, kanamycin 30 μg, nalidixic acid 30 μg, streptomycin 10 μg, tetracycline 30 μg, and trimethoprim/sulfamethoxazole 25 (1.25+23.75) μg.

Susceptibility or resistance was interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2008) guidelines and minimum inhibitory concentrations (MIC) for nalidixic acid and ciprofloxacin were determined by E-test on Mueller-Hinton agar following the manufacturer's instructions (AB-Biodisk, Solna, Sweden). E-tests were also performed in the presence of the efflux pump inhibitor phenylalanine-arginine-β-naphthylamide, which was added to Mueller-Hinton agar plates at a concentration of 20 mg/L. *Escherichia coli* ATCC™25922 was used for quality-control purposes.

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Table 1. Bacterial Isolates, Their Corresponding Bio/Serotypes and Minimum Inhibitory Concentrations (MICs) for Nalidixic Acid and Ciprofloxacin in the Presence and Absence of the Efflux Pump Inhibitor, Phenylalanine-Arginine-β-Naphthylamide (PAβN)

Bacterial isolate	*Bio/serotype*	*MIC (mg/L)*			
		NA	*NA + PAβN*	*CIP*	*CIP + PAβN*
YE 8081	1B	>256	32 (8)	0.25	0.19 (1)
506-06	4:O3	>256	48 (5)	0.25	0.19 (1)
871-07	1A	>256	32 (8)	1	0.38 (3)
10-2307	4:O3	2	0.125 (16)	0.03	0.02 (1.5)
ATCC™ 9610	1:O8	0.75	0.19 (4)	0.004	0.004

Values represent mean results from three independent determinations. Numbers in parenthesis represent the fold-reduction in MIC in the presence of the efflux pump inhibitor.

YE, *Yersinia enterocolitica*; NA, nalidixic acid; CIP, ciprofloxacin. Shaded blocks indicate resistant values.

Detection of virulence markers and the Y. enterocolitica*–specific 16S rRNA gene by polymerase chain reaction (PCR)*

Template DNA was prepared and three specific primer sets (Eurofins MWG Operon, Ebersberg, Germany) were used to amplify *ail* (Falcão *et al*., 2004), p*Yad* (Lantz *et al*., 1998), and the *Y. enterocolitica*–specific chromosomal 16S rRNA genes (Murphy *et al*., 2010). Amplification reactions were performed as described previously (Murphy *et al.,* 2010).

PCR amplification and DNA sequence analysis of QRDR and PMQR

QRDR containing regions of the target genes were amplified by PCR. Primer sequences and PCR conditions applied in 50-μL final reaction mixtures are shown in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/fpd). DNA sequences were analyzed for mutations using DNAStar software (Madison, WI), BLAST (http://blast.ncbi.nlm.nih.gov/), and ClustalW (http://www.ebi.ac.uk/clustalw). Similarly, PMQR primers and amplification conditions are shown in Supplementary Table S2. All amplicons produced were separated by electrophoresis in a 1.5% agarose gel (SeaKem® LE Agarose, Lonza Wokingham, Ltd., UK) containing 0.1 μg/mL EB (Sigma, Ireland) in 1 X Tris–boric acid–EDTA buffer (pH 8) (Sigma).

Table 2. Amino Acid Substitutions Identified Within the Quinolone-Resistance-Determining Regions of DNA Gyrase

Bacterial isolate	*GyrA*
YE 8081	Asp87→Tyr
506-06	Asp87→Tyr
871-07	Ser83→Ile
10-2307	none

YE, *Yersinia enterocolitica.*

Results and Discussion

The three *Y. enterocolitica* strains were isolated from humans between 2006 and 2010, in Switzerland. Two of the isolates were bio/serotypes 4:O3 (506-06 and 10-2307) and are classified as pathogenic. The remaining isolate was of biotype 1A (871-07). Isolates 506-06 and 10-2307 harbored the large pVYe plasmid of 67-kb (data not shown) and were positive for the *ail* and p*Yad* markers, features associated with pathogenic *Y. enterocolitica* bio/serotypes (Lantz *et al.,* 1998; Falcão *et al.,* 2004; Thisted Lambertz *et al.,* 2006). Isolate 871-07 did not contain this plasmid, consistent with the lack of *ail* and p*Yad* markers, and the 1A biotype.

All three clinical isolates were resistant to nalidixic acid by disk diffusion with resistance profiles including 506-06: AmpKfNaSSxt; 871-07: AmcAmpKfNa; 10-2307: AmpKfNa. Two of the three clinical isolates (506-06 and 871-7, Table 1) were resistant to nalidixic acid with MIC > 256 mg/L. All three clinical isolates were susceptible to ciprofloxacin and none contained PMQR markers (data not shown).

The amino acid substitutions associated with nalidixic acid resistance are summarized in Table 2. A mutation in *gyrA* was identified in isolate 871-07 consistent with the substitution of Ser-83-Ile, and an Asp-87-Tyr substitution was identified in isolate 506-06, similar to the change in *Y. enterocolitica* 8081. Mutations in these loci contributed to a nalidixic acid–resistant phenotype, as determined by E-test (Table 1). Furthermore, no mutations in *gyrB, parC*, or *parE* were detected.

Measurement of MICs to nalidixic acid and ciprofloxacin in the presence of phenylalanine-arginine-β-naphthylamide contributed to an increase in susceptibility with the exception of the reference strain, in respect of ciprofloxacin (Table 1). These findings suggest that efflux activity in this bacterium contributes to quinolone/FQ resistance, a feature that confirms earlier observations (Capilla *et al*., 2004).

Compared to other Enterobacteriaceae, small numbers of *Y. enterocolitica* resistant to nalidixic acid have been reported to date (Sánchez-Céspedes *et al*., 2003; Capilla *et al*., 2004; Fàbrega *et al*., 2010; Sihvonen *et al*., 2011; Fredriksson-Ahomaa *et al.,* 2012). Although target gene mutations in *gyrA* are the primary mechanism of resistance to nalidixic acid, efflux pump activity, also contributes to support this phenotype. This finding supports data reported earlier (Capilla *et al*., 2004). Importantly, all three clinical isolates remain susceptible to ciprofloxacin.

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Disclosure Statement

No competing financial interests exist.

References

Capilla S, Ruiz J, Goñi P, Castillo J, Rubio MC, Jiménez de Anta MT, Gómez-Lus R, Vila J. Characterization of the molecular mechanisms of quinolone resistance in *Yersinia enterocolitica* O:3 clinical isolates. J Antimicrob Chemother 2004;53:1068–1071.

Cattoir V, Poirel L, Rotimi V, Soussy CJ, Nordmann P. Multiplex PCR for detection of plasmid-mediated quinolone resistance

qnr genes in ESBL-producing enterobacterial isolates. J Antimicrob Chemother 2007a;60:394–397.

Cattoir V, Weill FX, Poirel L, Fabre L, Soussy CJ, Nordmann P. Prevalence of *qnr* genes in *Salmonella* in France. J Antimicrob Chemother 2007b;59:751–754.

Cavaco LM, Hasman H, Xia S, Aarestrup FM. *qnr*D, a novel gene conferring transferable quinolone resistance in *Salmonella enterica* serovar Kentucky and Bovismorbificans strains of human origin. Antimicrob Agents Chemother 2009;53: 603–608.

[CLSI] Clinical Laboratory Standards Institute. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals*; *Approved Standard*, 3rd ed. CLSI Document M31-A3. Wayne, PA: CLSI, 2008.

Drummond N, Murphy BP, Ringwood T, Prentice MB, Buckley JF, Fanning S. *Yersinia enterocolitica:* A brief review of the issues relating to the zoonotic pathogen, public health challenges and the pork production chain. Foodborne Pathog Dis 2012;9:179–189.

Fàbrega A, Roca I, Vila J. Fluoroquinolone and multidrug resistance phenotypes associated with the overexpression of AcrAB and an orthologue of MarA in *Yersinia enterocolitica*. Int J Med Microbiol 2010;300:457–463.

Falcão JP, Brocchi M, Proenca-Modena JL, Acrani GO, Correa EF, Falcão DP. Virulence characteristics and epidemiology of *Yersinia enterocolitica* and *Yersiniae* other than *Y. pseudotuberculosis* and *Y. pestis* isolated from water and sewage. J Appl Microbiol 2004;96:1230–1236.

Fredriksson-Ahomaa M, Cernela N, Hächler H, Stephan R. *Yersinia enterocolitica* strains associated with human infections in Switzerland 2001–2010. Eur J Clin Microbiol Infect Dis 2012;31:1543–1550.

Karczmarczyk M, Martins M, Quinn T, Leonard N, Fanning S. Mechanisms of fluoroquinolone resistance in *Escherichia coli* isolates from food-producing animals. Appl Environ Microbiol 2011;77:7113–7120.

Lantz PG, Knutsson R, Blixt Y, Abu Al-Soud W, Borch E, Radostrom P. Detection of pathogenic *Yersinia enterocolitica* in enrichment media and pork by a multiplex PCR: A study of sample preparation and PCR-inhibitory components. Int J Food Microbiol 1998;45:93–105.

Murphy BP, Drummond N, Ringwood T, O'Sullivan E, Buckley JF, Whyte P, Prentice MB, Fanning S. First report: *Yersinia enterocolitica* recovered from canine tonsils in Ireland. Vet Microbiol 2010;146:336–339.

Park CH, Robicsek A, Jacoby GA, Sahm D, Hooper DC. Prevalence in the United States of *aac(6′)-Ib-cr* encoding a ciprofloxacin-modifying enzyme. Antimicrob Agents Chemother 2006;50:3953–3955.

Ruiz J. Mechanisms of resistance to quinolones: Target alterations, decreased accumulation and DNA gyrase protection. J Antimicrob Chemother 2003;51:1109–1117.

Sánchez-Céspedes J, Navia MM, Martínez R, Orden B, Millán R, Ruiz J, Vila J. Clonal dissemination of *Yersinia enterocolitica* strains with various susceptibilities to nalidixic acid. J Clin Microbiol 2003;41:1769–1771.

Sihvonen LM, Toivonen S, Haukka K, Kuusi M, Skurnik M, Siitonen A. Multilocus variable tandem-repeat analysis, pulsed-field gel electrophoresis, and antimicrobial susceptibility patterns in discrimination of sporadic and outbreak-related strains of *Yersinia enterocolitica*. BMC Microbiol 2011;11:42e.

Thisted Lambertz S, Granath K, Fredriksson-Ahomaa M, Johansson KE, Danielsson-Tham ML. Evaluation of a combined culture and PCR method (NMKL-163A) for detection of presumptive pathogenic *Yersinia enterocolitica* in pork products. J Food Prot 2006;70:335–340.

Wang M, Guo Q, Xu X, Wang X, Ye X, Wu S, Hooper DC, Wang M. New plasmid-mediated quinolone resistance gene, *qnrC*, found in a clinical isolate of *Proteus mirabilis*. Antimicrob Agents Chemother 2009;53:1892–1897.

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